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Cell-Mediated Mutagenesis, an Approach to Studying Organ Specificity of Chemical Carcinogens

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16. ABSTRACT

A number of approaches have been employed to elucidate the causes of organ and species specificity (8-15). In the earlier studies, homogenates of various tissues were used to metabolically activate the chemical (9-12); however, metabolite profiles, DNA-adduct profiles, and induced mutagenic responses of cell homogenates differ from those of intact cells (16-19). More recent studies have looked at the nature of carcinogen metabolites and DNA-adducts formed in cells or tissues or at the nature and persistence of DNA-adducts in in vivo tissues (15). Differences in the results from these various approaches are apparent. In addition, the nature and amount of DNA-adducts are only correlated with the biological response expected in the tissue. The ability to measure a biological response from a system as well as parameters such as chemical metabolism, DNA adducts, DNA repair, etc., would provide a useful system for elucidating some of the biological mechanisms leading to the phenomena of specificity.

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CELL-MEDIATED MUTAGENESIS, AN APPROACH TO STUDYING ORGAN SPECIFICITY OF CHEMICAL CARCINOGENS

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INTRODUCTION

We have been interested in developing in vitro systems in which the organ and species specificity of chemical carcinogens can be studied (1-6). The phenomena of species, organ, sex, and age specificity in response to chemical carcinogens pose an intriguing problem and may be important for the extrapolation of in vivo and in vitro carcinogenesis data to humans. Many carcinogens exist in the environment in nonreactive form and must be metabolically activated to manifest their biological activity (7,8).

A number of approaches have been employed to elucidate the causes of organ and species specificity (8-15). In the earlier studies, homogenates of various tissues were used to metabolically activate the chemical (9-12); however, metabolite profiles, DNA-adduct profiles, and induced mutagenic responses of cell homogenates differ from those of intact cells (16-19). More recent studies have looked at the nature of carcinogen metabolites and DNA-adducts formed in cells or tissues or at the nature and persistence of DNA-adducts in in vivo tissues (15). Differences in the results from these various approaches are apparent. In addition, the nature and amount of DNA-adducts are only correlated with the biological response expected in the tissue. The ability to measure a biological response from a system as well as parameters such as chemical metabolism, DNA adducts, DNA repair, etc., would provide a useful system for elucidating some of the biological mechanisms leading to the phenomena of specificity.

In the approach described here, intact cells are obtained from adult male rat liver, lung, bladder, and kidney by enzymatic

dispersion, and the cells are cocultivated with Chinese hamster V79 cells in the presence of the chemical (1-6). The mutation of the V79 cells from ouabain or 6-thioguanine (TG) sensitivity to resistance then indicates the production of mutagenic/carcinogenic metabolites of the chemical. The V79 cells alone cannot activate the chemicals being studied; therefore, the production of mutagenic intermediates reflects the specificities of activation of the different primary cell types. By using this approach, factors other than metabolism believed to be important in determining organ or species specificities (24) such as DNA repair, nature of DNA-adducts, cell uptake of the chemical, etc., can also be studied and correlated with the mutagenesis endpoint.

In the present report, carcinogens from three classes of chemicals were studied for mutagenic activity with the primary cells from the four organs: liver, lung, kidney, and bladder. The metabolism of the carcinogen benzo[a]pyrene (BP) was studied in detail in all cell systems by high pressure liquid chromatography (HPLC), while nitrosodimethylamine (DMN) metabolism was investigated in the liver and lung system. An attempt was made to correlate metabolism with mutagenic activities and also to correlate cell-mediated mutagenesis data with *in vivo* carcinogenesis data.

MATERIALS AND METHODS

Six- to ten-week-old male Sprague-Dawley rats were the source of the primary liver, lung, kidney, and bladder cells. Methods for preparing liver, lung, and bladder cells were reported previously (1-4). Kidney cells were dissociated enzymatically for 1.5 h at 37°C from minced kidney tissue with 0.13% trypsin and 0.01% EDTA containing 50 units/ml of collagenase. All primary cell types were more than 90% viable at the time of seeding. The medium used was Williams Medium E plus 10% heat-inactivated fetal bovine serum supplemented with 2 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were maintained at 37°C in humidified incubators with an atmosphere of 5% CO_2 in air.

The protocols for the mutation studies were reported previously (1-4). The background mutation frequency was 1 mutant/10^6 survivors for ouabain and <3 mutants/10^6 survivors for TG. The expression time was 2 days for ouabain resistance and 6 days for TG (2,6).

The BP and DMN metabolism studies were carried out as previously reported (2).

RESULTS

The basic protocols for carrying out the cell-mediated mutagenesis studies were reported previously (1-6). Figure 1 shows schematically how the hepatocyte or bladder cell-mediated V79 cell mutagenesis systems are performed. Due to slower attachment, the lung and kidney cells are seeded first and allowed to attach, followed by the V79 cells. In all the systems, however, the ratio of activating cells to V79 cells is about 3:1 or 4:1. Using the hepatocyte system as an example (see Figure 1), the hepatocytes attach on top of the V79 cells, forming a near monolayer. The medium is then changed to fresh medium containing the chemical, and the cocultivation is continued for 48 h. The cells are then reseeded to determine toxicity and mutation to the V79 cells. The activating cells do not reattach and grow in this procedure. In this system, the liver cell metabolizes the chemical to a reactive intermediate that is then transported, presumably via cell-to-cell junctions to the V79 cells, which are mutated. A proximity between the activating cell and the target cells may be a prerequisite for the occurrence of mutagenesis, although the more stable the intermediate or reactive form, the greater the distance it can transverse.

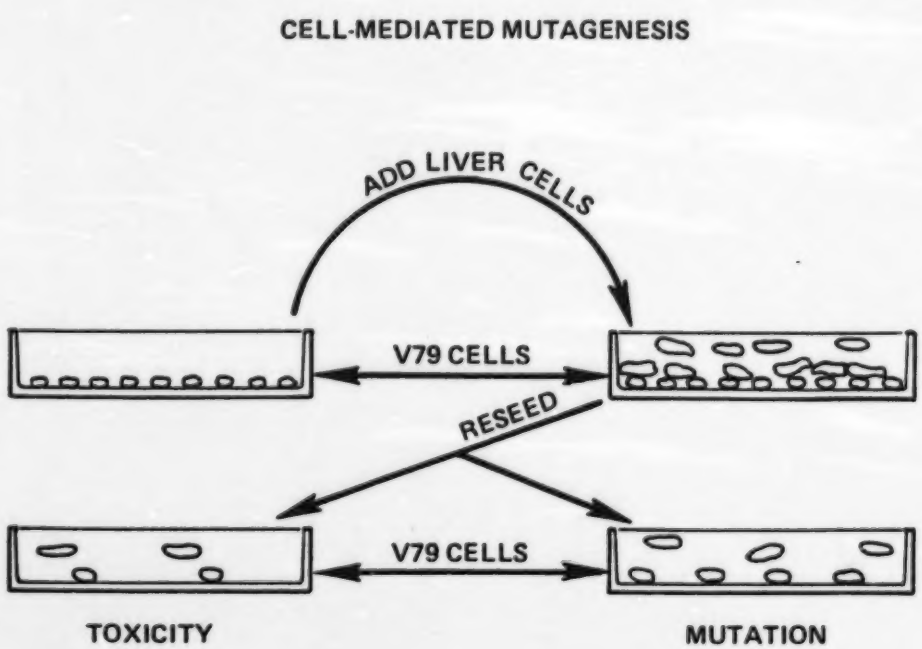

Figure 1. Diagram of cell-mediated mutagenesis protocol.

Interestingly, the hepatocyte to V79 cell arrangement shown in Figure 1 may be an *in vitro* model for the events that occur *in vivo* during the initiation of transformation or mutation. In other words, the differentiated cell (hepatocyte, in this instance) has the metabolic capabilities to carry out the tissues' metabolic functions, but lacks or has limited replicative capabilities. The less well differentiated cells, including stem cells (represented by the V79 cells) that possibly have less metabolic capabilities but can replicate, are arranged in proximity to the differentiated cells. Therefore, *in vivo*, the terminally differentiated cells metabolize the xenobiotic, and the activated intermediates are transported to the stem cells. The mutated or transformed stem cells on replication could give rise to the mutant colony or tumor. Such a phenomenon could also explain the long known difficulty in mutating or transforming differentiated cells from adult animal tissues *in vitro*.

The data in Table 1 summarize our initial studies to investigate the cell type specificity in the activation of chemical carcinogens. BP, a potent skin and lung carcinogen capable of also producing fibrosarcomas, and aflatoxin B_1 (AFB_1), a potent liver carcinogen, were studied for mutagenic activity in the rat embryonic fibroblast- and the adult rat hepatocyte-mediated mutagenesis system. BP was mutagenic in the embryonic fibroblast-mediated system, while in the hepatocyte-mediated system, no significant increase in mutagenic activity was observed with up to 3 μg/ml BP. In contrast, AFB_1 was not mutagenic in the fibroblast-mediated system but was mutagenic in the hepatocyte-mediated system. These studies established that a cell type specificity could be observed *in vitro*, which agreed with the *in vivo* tumorigenic response of the two cell types.

Table 1. Mutagenic Activities of BP and AFB_1 in the Hepatocyte- and Embryonic Fibroblast-Mediated Mutagenesis System

Activating Cell	Chemical	Mutants/10^6 Survivors
None	--	1
Liver	BP	2
Liver	AFB_1	39
Fibroblasts	BP	96
Fibroblasts	AFB_1	2

We had two major concerns about this initial study. First, we were comparing mixed embryonic cells to cells from an adult animal organ. Secondly, fibroblasts were being compared to epithelial cells for activation potential, while the majority of tumors in man and animals arise in the epithelium of various organs. In an attempt to overcome the limitations of our initial approach, we began to develop a method for conducting cell-mediated mutagenesis studies with epithelial cells from various adult tissues (1,2,5,6). The data in Table 2 show the relative mutagenic activity of three potent chemical carcinogens in the liver, lung, bladder, and kidney cell-mediated mutagenesis systems. All of these studies used primary cultures of intact cells from the respective organs, and the cultures were predominantly of epithelial-like cells. As can be seen in Table 2, the noncarcinogenic hydrocarbon anthracene was not mutagenic in any of the cell-mediated systems. In the liver cell-mediated system, BP was inactive as indicated above, while 7,12-dimethylbenz[a]anthracene (DMBA) was somewhat mutagenic, and DMN was highly mutagenic in this system. By contrast in the lung cell-mediated system, the two hydrocarbons were mutagenic, while DMN was inactive. The kidney cells were able to activate the two hydrocarbons and the nitrosamine. Likewise, cells from the bladder epithelium activated all three carcinogens. The data in Table 2 are expressed as fold increase over background per 1.5×10^6 activating cells. For the liver and lung cell systems, ouabain, which gave a background of 1 mutant/10^6 surviving V79 cells (2), was used as the selective agent. However, for the kidney and bladder cell-mediated systems (6), TG was used as the selective agent, and the spontaneous frequency was 3×10^6 mutant/10^6 surviving V79 cells. We reported previously that by applying this method for certain chemicals, the relative activities can be compared even when different selective agents are used (19). However, the generality of this approach has yet to be determined.

Table 2. Relative Mutagenic Activities in the Rat Cell-Mediated Mutagenesis Systems

		Activating Cell Type			
Chemical	(μg/ml)	Liver	Lung	Kidney	Bladder
Anthracene	(3)	1	1	1	1
BP	(1)	2	56	5	6
DMBA	(1)	14	154	12	21
DMN	(100)	95	2	19	11

Having determined the mutagenic activities of the carcinogens in the various cell-mediated systems, we looked at carcinogen metabolism to determine if correlations between metabolism and mutagenesis could be discerned. The metabolism of BP was accomplished by HPLC analysis, and the metabolism of DMN was determined by CO_2 production (2).

The HPLC profiles of BP metabolism for primary cells were reported previously (1,2,6). All cell lines produce metabolites that are organically soluble or are conjugated to glucuronic acid or sulfate (2,6). The data in Table 3 show the amounts of three types of metabolites produced by the primary cells in culture. These particular metabolites were chosen for discussion here because of their potential involvement in the transformation or mutation process. The pre-9,10-diols constitute the early eluting fraction from the HPLC and presumably contain tetrols, triols, and other oxygenated metabolites, some of which probably at one stage in their metabolism were intermediates that could have reacted with cellular macromolecules. The BP-4,5-diol probably results from the BP-4,5-oxide, a species also capable of interacting with cellular constituents, although the 4,5-diol itself is inactive or weakly active as a mutagen to V79 cells (19). The BP-7,8-diol is a precursor to the BP-7,8-diol-9,10-epoxide, the presumed active metabolite of BP known (20). As can be seen in Table 3, the order of pre-9,10-diol production is lung > liver > bladder > kidney, which also is the order of total BP metabolism, including conjugates. The liver produces the greatest amount of the BP-4,5-diol. The BP-7,8-diol, thought to be a precursor to the most important biological metabolite, is formed mostly in the lung, the cell type where BP was also most mutagenic. Bladder and kidney cells, which also activate BP to mutagenic intermediates, produce significant levels of the 7,8-diol. However, liver cells, which do not activate BP to mutagenic intermediates, also produced the 7,8-diol. Therefore, the absolute amount of the BP-7,8-diol produced does not directly correlate with the mutagenic response in the cell-mediated systems.

The data in Table 4 show the metabolism of DMN by liver and lung cells. The DMN metabolism studies were carried out with cells from the two tissues immediately after their isolation, so that loss of enzyme capability due to isolation should have been minimized. DMN was metabolized to CO_2 by the liver cells (Table 4). However, lung cells, which did not metabolize DMN to mutagenic intermediates, also did not metabolize the DMN to detectable levels of CO_2. The lack of DMN metabolism by lung cells may account for its lack of mutagenicity in the lung cell-mediated system.

Table 3. BP-Diol Metabolites Formed by Liver, Lung, Bladder, and Kidney Cells

	pmoles Formed/48 h/10^6 Cells		
Cell Type	Pre-9,10-diol	4,5-diol	7,8-diol
Lung	4870	90	750
Liver	2320	217	212
Bladder	2655	108	415
Kidney	765	50	115

Table 4. Metabolism of DMN to CO_2 by Liver and Lung Cells

	DPM/10^6 Cells	
Time (h)	Liver	Lung
3	15,000[a]	ND[b]
6	41,000	ND

[a]DMN at 100 µg/ml, data approximately 1 and 3% metabolism at the respective times.
[b]ND, not detectable.

The data in Table 5 indicate that the cell-mediated mutagenesis approach can also be used to study species differences in the activation of chemical carcinogens. The data indicate that hamster hepatocytes are between three and four times as active at metabolizing DMN to mutagenic intermediates as are rat hepatocytes.

Table 5. Species Differences in the Activation of DMN to Mutagenic Intermediates[a]

Species	Mutants/10^6 Survivors
Control	1
Rat	95
Hamster	320

[a]DMN was used at 100 µg/ml.

DISCUSSION

The data reported herein suggest that the cell-mediated mutagenesis approach may be useful for studying some aspects of the organ and species specificity of chemical carcinogens. In Table 6 we compared the relative carcinogenic activities of the chemicals to their relative mutagenic activities in the cell-mediated system. As can be seen in Table 6, there is general agreement in the activities between the *in vivo* and *in vitro* results. The relative carcinogenic activities for liver, lung, and kidney, was assigned from literature references (21-24); rodent bladder carcinogenesis data with these chemicals are limited. DMBA has been reported to cause focal hyperplasia and papillomas in the bladder (21) and to transform bladder epithelial cells in organ culture (25). The finding that bladder epithelial cells can activate DMN is important, given its presence in urine of humans with certain types of urinary tract infections (26). While DMN has not been shown to induce bladder cancer in animals or humans, people with frequent urinary tract infections have an increased incidence of bladder cancer (27). BP also has not been shown to induce bladder cancer, although correlations between bladder cancer and smoking have been shown (28), and BP, along with other known carcinogens, is a component of cigarette smoke. The possible involvement of BP glucuronides in bladder cancer has been suggested (29). The present studies predict that these carcinogens may induce bladder cancer and demonstrate the role of the bladder epithelium in the activation of some chemical carcinogens.

Because of the differential mutagenic responses to BP and DMN in the various organs, the metabolism of these carcinogens in the primary cells was undertaken. All the cell types are capable of metabolizing BP, although quantitative differences in the total metabolism and individual metabolites existed. Glucuronidation and formation of sulfates of BP also occurred in all tissues. However, as stated above, a precise correlation between any metabolite or

Table 6. Comparison of Relative Carcinogenic and Mutagenic Activities[a]

	Target Organ or Activating Cell Type							
	Liver		Lung		Kidney		Bladder	
Chemical	Car.	Mut.	Car.	Mut.	Car.	Mut.	Car.	Mut.
BP	±	-	++	++	+	+	-	+
DMBA	+	+	++	++	++	+	±	+
DMN	++	++	±	-	++	+	?	+

[a]Mutation: -, <3 x background;
+, 4 to 25 x background;
++, >25 x background.

or pattern of metabolites and mutagenic activity could not be discerned. This finding again emphasizes that metabolism is one of many factors leading to organ and species specificity.

A major concern in developing in vitro systems to study various processes involved in carcinogenesis is how well the in vitro results simulate the in vivo situation. The possible similarity of the cell-mediated approach to in vivo cell interaction has been depicted in Figure 1. When carrying out the cell-mediated mutagenesis approach, several factors must be considered. A possible limitation of using intact cells for activation is whether the activated metabolite can transverse the distance to the DNA of the V79 cell. This transportability depends in part on the stability of the active intermediate and/or whether the proper junctions (if needed) form between the metabolizing cell and V79 cell during the short coculture time. A proximity between the activating cell and target cell has been reported to be essential (30); the extent of cell-to-cell closeness required probably will vary with the stabilities of the active intermediates. At this time, information concerning this aspect is limited, and further studies in these areas would be informative and useful. The data do indicate that several classes of carcinogens can be assayed in the cell-mediated system with various primary activating cell types. Another concern about the cell-mediated approach is the possible selection for or against

certain cell types during the tissue disssociation and seeding procedures. Those cell types that are only a small proportion of the total tissue but are major sources of tumors may not be adequately represented for activation capability in the dissociated cell populations. Most of these limitations can be investigated and possibly overcome by improved cell culture methodologies and specific cell type isolation techniques. Pharmacodynamics and tissue distribution of the carcinogens are phenomena which may not be entirely simulated in the current approach. However, studies of relative cell uptake may aid in working this variable into the system.

A future worthwhile application of the cell-mediated approach would be the utilization of human cells from various tissues for metabolic activation and mutable or transformable human cells as the target cells. In this in vitro all human cell system, one would be studying directly the effect of the chemical being applied to human tissues. Concommitant with this approach, the other biological parameters (i.e., DNA-adducts, etc.) could be measured. This battery of biological data then could be used to determine the target organ and potential risk of the agent to man. The accumulation of such data may provide a method for extrapolating risk to humans that would not involve extrapolation directly from in vivo rodent studies. Such an approach appeals, because it does not depend on the long-term maintenance of differentiated human cells in culture, a difficult task to date. Thus, the potential utilization of the cell-mediated approach appears to have fundamental as well as applied uses in chemical carcinogenesis.

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DISCUSSION

Q.: You told us earlier on that you had the feeling that the cooperation between the metabolizing cells and the mutable cells might be important. Then you showed us that picture of the very few bladder cells lying on top of an awful lot of these 79 cells. Have you tried any experiments to see whether the mutagenic potency will increase as you approach a monolayer or near monolayer of bladder cells on top of your V79 cells?

A.: Yes, we have. There wasn't time to point this out, but we have conducted both dose responses with all the chemicals and cell number responses with all cell types. When we carry out the mutagenesis experiments, we usually pretty nearly have a monolayer of these bladder cells on top of the V79 cells. We find that a ratio of about four activating cells per V79 cell is about optimal.

Q.: Just a couple of comments regarding your tables. One is with DMBA in the bladder system. DMBA in vitro has been shown to transform bladder cells in organ culture. The other is for the kidney. The kidney is not just a kidney. It's a combination of at least seven organs, probably even more, and to just throw in a kidney cell is not going to give you the kind of answers you're looking for, particularly when you need to separate cortex and medulla.

A.: Your points are very well taken. In the organ culture studies, I don't recall which cell types were transformed. If they were fibroblast or the transformed cells gave rise to fibrosarcomas, the system is not as meaningful as if epithelial cells were transformed and carcinomas produced. In our system, we have predominantly epithelial cells for activation and I consider this significant.

Regarding the kidney, it is our goal to separate it into various cell types.

Q. I would just like to follow up on that comment. In vivo, one would assume that these carcinogens must pass through the endothelium (which is a major cell type in the lung), and I was wondering if you had looked at isolated endothelial cells.

A. No, we haven't as yet isolated individual lung cell types either, but in our lung population we do have endothelial cells in our lung cell preparations when we do the cell-mediated studies.